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Tracing Photosynthetic Response Curves with Internal CO_2 Measured Directly

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In this study, a system to measure leaf internal CO_2 (C_i) was incorporated into an open gas exchange system (LI-COR, Lincoln, NE, USA). The C_i was directly measured with a cup attached to the abaxial surface of sunflower (*Helianthus annuus* L.) leaves with open stomata while normal CO_2 and water vapor exchange through the same section of adaxial surface was simultaneously detected. The potential problems in the system, namely bulk air flow through the leaf, diffusion leaks, and change in the CO_2 gradient inside the leaf, were examined with the aim to apply the system to measure net photosynthesis at various C_i (i.e. $A-C_i$ curves). A micro blower constantly circulated the air in a loop without pressure pulses or bulk air movement through the leaf. The measured C_i ($C_{i(m)}$) generally followed the external CO_2 as much as the calculated one ($C_{i(c)}$). There was close agreement between the $C_{i(m)}$ and the $C_{i(c)}$ particularly at low C_i, and the diffusion leak hardly affected the relationship between the two. Despite possible alterations of leaf properties by cup attachment, the direct measurement is expected to cast a new light on leaf gas exchange.

Keywords : A–C_i curve, amphistomatous, gas diffusion, *Helianthus annuus* L., internal CO_2 gradient, LI-6400

INTRODUCTION

For photosynthesis, CO_2 is critical as a substrate for the reaction. The CO_2 diffuses from the outside to the intercellular airspace through stomatal pores on the surface of the leaf. From there, it diffuses in the liquid phase to the fixation sites in the mesophyll cells (Evans and Loreto, 2000). Diffusion is a passive physical process, but in plants can be regulated. Stomatal closure is the most direct means of regulation, and closure prevents excessive cellular water loss. But inward CO_2 diffusion is physically restricted because water vapor and CO_2 share common diffusion pathways via the stomata. Consequently, closure leads to a decrease in the internal CO_2 concentration (C_i) and rate of assimilation (A).

The photosynthetic CO_2 response curve, namely $A-C_i$ curve, has been widely used to assess the photosynthetic capacity because it eliminates gas phase diffusion or stomatal conductance (g_s) in which changes in the curve are considered indicators of non-stomatal limitation on photosynthesis (Boyer, 1971; Farquhar and Sharkey, 1982; Graan and Boyer, 1990). In most studies, C_i is routinely calculated from the outward diffusive behavior of water vapor (Moss and Rawlins, 1963; Jarman, 1974; von Caemmerer and Farquhar, 1981). When stomata are open, the calculation appears reasonably accurate (Sharkey et al., 1982). Sharkey et al. (1982) measured C_i directly along with the standard gas exchange parameters. Later, Boyer and Kawamitsu (2011) incorporated the C_i measurement of Sharkey et al. (1982) into a gas exchange system. They experimentally measured the $A-C_i$ curve in sunflower, determined the effect of stomatal closure, and confirmed the hindrance of water vapor on entry of CO_2.

Direct C_i measurement has a potential advantage when stomata close, which increases the cuticle influence (Boyer et al., 1997; Meyer and Genty, 1998) or patchiness of stomatal closure (Terashima et al., 1988; Mott, 1995; Buckley et al., 1997). These are considered problems for the $A-C_i$ analysis, which usually depends on calculated C_i and thus the diffusive behavior of water vapor. Lauer and Boyer (1992) and Boyer and Kawamitsu (2011) considered the direct method to be a robust approach for tracing $A-C_i$ curves. In this study, we incorporated a direct C_i measurement system into the LI-6400 open gas exchange system (LI-COR, Lincoln, NE, USA). The applicability of the system for $A-C_i$ curve measurement was tested in the sunflower (*Helianthus annuus* L.) leaf. We took special care to minimize bulk air flow through the leaf (Lauer and Boyer, 1992; Boyer and Kawamitsu, 2011), diffusion leaks (Flexas et al., 2007; Rodeghiero et al., 2007), and changes in the CO_2 gradient across the leaf mesophyll (Mott and O'Leary, 1984; Parkhurst et al., 1988; Parkhurst and Mott, 1990) that can be important for the measurement.

MATERIALS AND METHODS

Plant material

Sunflower (*Helianthus annuus* L.) plants were grown in a glasshouse located in the Department of Agriculture, University of the Ryukyus, Okinawa, Japan (26°15′N, 127°45′E; altitude 127 m). In December 2013, seeds were germinated in a fertilized seeding soil with 380, 290 and 340 mg l^{-1} of N:P:K (Takii & Co., Ltd., Kyoto, Japan). After

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10 d, seedlings were transplanted and grown in 4-L plastic pots containing a soil mixture consisting of 1:1:1 soil:peat:sand. The plants were automatically watered three times each d and were fertilized weekly with 500 ml of Hoagland's nutrient solution. Fluorescent light was supplemented when photosynthetic photon flux density (PPFD) above the plants fell below 800 μmol m^{-2} s^{-1}. Daylength in the glass-house was extended to 15 h to prevent flowering. The day and night temperatures ranged from 17–24 °C and 13–22°C, respectively. Only upper fully expanded leaves (130–180 cm^2) from 7–8 weeks old plants were used.

Gas exchange systems

To measure internal CO_2 concentration directly, we made a small acrylic chamber (cup) which can be incorporated into a commercially available open gas exchange system (LI-6400XT; LI-COR, Lincoln, NE, USA). The cup was specially designed for the bottom half of an integrated fluorescence chamber head (LI-6400-40; LI-COR), having a round airspace with 2 mm depth surrounded by the black neoprene gasket (6400-41; LI-COR) which shares the same leaf area (2 cm^2) with the upper half (Fig. 1). In the cup, the CO_2 equilibrated with that in the stomatal pores adjacent to the airspace (Sharkey et al., 1982; Lauer and Boyer, 1992; Boyer and Kawamitsu, 2011). The cup was connected in a closed loop with an IRGA (LI-840A; LI-COR) and a micro blower (109P0412H309; Sanyo Denki Co., Ltd., Osaka, Japan) which allows the air to gently circulate around the loop (ca. 300 ml min^{-1}) (Fig. 2). The water jacketed Y- shape glass tube (condenser) connected in the loop and a bubble seal to its lower end ensured atmospheric pressure and free of condensation in the loop path and the cup. Pressures in the loop were also monitored continuously in the optical cell of the Li-840A. The blower generated a pressure differential of about 0.04 kPa without any pulses. The condensation occurred slowly in the condenser but not the other parts of tubing or cup. The condensate naturally dripped through the inner wall of the condenser to the surface of the water seal and did not affect the measurement. The cup was located opposite the blower in the closed loop so as to prevent bulk air movement through the leaf (Boyer and Kawamitsu, 2011). The approximate total volume of the closed system was 100 ml (including LI-840A) with a total path length of 1.8 m. Leaf temperature was measured with a fine 0.13 mm chromel-constantan thermocouple (CHCO-005; Omega engineering, Stanford, CT, USA) appressed to the underside of the leaf by the flexed stainless wire in the cup (Fig. 1). The sensor was connected to the chamber head so that the leaf temperature was led to the LI-6400 console in the usual manner. While the bottom cup measured CO_2 equilibrated with that in the intercellular spaces of the leaf ($C_{i(m)}$) the upper half measured standard gas exchange parameters including calculated internal CO_2 ($C_{i(c)}$). In the LI-6400 gas exchange system, the correction of the two IRGAs (for reference/ sample), known as 'match', is essential for the measurement precision. We retained this feature by bypassing the cup to the exhaust, which enabled the two IRGAs to be matched dur-

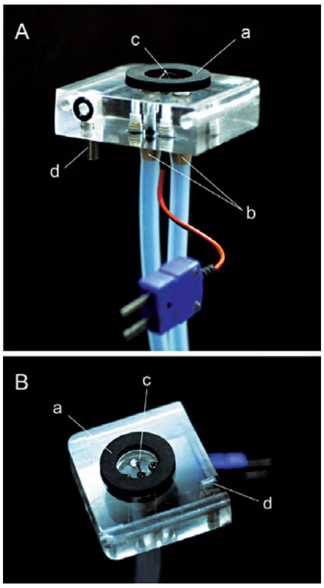

Fig. 1 The cup for direct measurement of internal CO_2 (C_i). View from the side (A) and from above (B). The cup was specially designed for the bottom half of an integrated fluorescence chamber head (LI-6400-40; LI-COR). The black neoprene gasket (a; 6400-41; LI-COR) surrounds a round airspace with 2 mm depth, and shares the same leaf area (2 cm^2) with the upper half chamber. The air was circulated through both inlet and outlet (b) located at the bottom of the cup. Leaf temperature was measured with a fine 0.13 mm chromel-constantan thermocouple (c; CHCO-005; Omega engineering, Stanford, CT, USA) appressed to the underside of the leaf by the flexed stainless wire in the cup. The bypass to the exhaust (d) allows matching the two IRGAs during measurements.

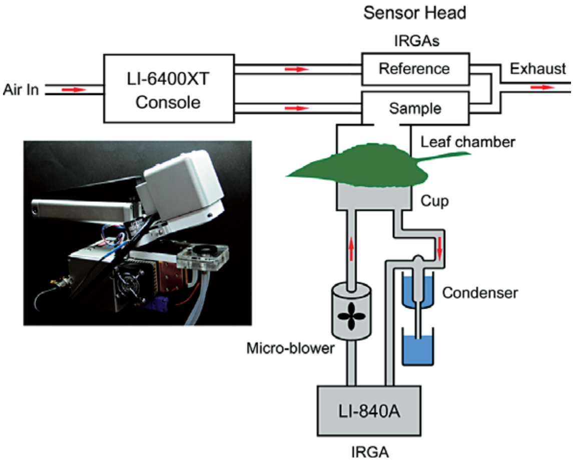

Fig. 2 Schematic diagram of the gas exchange system with internal CO_2 (C_i) directly measured. The closed loop for the direct measurement of C_i is shown in dark color, whereas the flow path of the LI6400XT (Li-Cor) open gas exchange system is in white color. The cup was connected in the closed loop with the IRGA (LI-840A; LI-COR) and the micro blower which allows the air to gently circulate around the loop (ca. 300 ml min^{-1}). The condenser ensured atmospheric pressure free of condensation in the loop path and the cup. The approximate total volume of the closed system was 100 ml (including LI-840A) with a total path length of 1.8 m.

ing measurements (Fig. 1). CO_2 concentration was regulated with pure CO_2 in a tank connected to the LI-6400 console and CO_2-free air primarily passed through soda lime. Humidity was controlled by a dew point generator (LI-610; LI-COR) with the CO_2-free air. We modified the system to attain low CO_2 concentration (<50 μmol mol^{-1}) according to LI-COR (2010). Both IRGAs for LI-6400 and LI-840A were calibrated using the same standard gases. For LI-6400 calibration was performed with 0 and 400 μmol CO_2 mol^{-1} air whereas for LI-840A additional 2000 μmol CO_2 mol^{-1} air was used for the higher CO_2 range.

Leak test

To test the diffusion leak in the closed loop, 0.2 ml of either 1 or 5% CO_2 was injected into the closed loop from the water seal of the condenser. Instead of a leaf an aluminum foil was clamped by the chamber to isolate the loop from the open gas exchange system. This amount of injection did not cause a detectable increase in pressure in the closed loop. The CO_2 injection was also conducted for intact leaves to examine the response of photosynthesis towards equilibration.

Diffusion leaks may also occur in an open gas exchange system (LI-COR, 2008). We estimated diffusion molar flow rate of CO_2 (K_{CO2}) and water vapor (K_{H2O}) according to Flexas et al. (2007) and Rodeghiero et al. (2007). Previous studies (Lauer and Boyer, 1992; Boyer and Kawamitsu, 2011) have preferentially used paraffin/-lanolin (P/L) coat to prevent diffusion leaks in the gas exchange systems. Accordingly, we tested the effects of the P/L (2:8) as well as Vaseline (Unilever, Rotterdam, The Netherlands) and a high vacuum grease (Dow Corning Toray Co., Ltd., Tokyo, Japan) on the leaks. The CO_2 and water vapor concentration outside the chamber was allowed to fluctuate but monitored during all the measurements by using an open path IRGA (LI-7500; LI-COR) set around the chamber head.

$A-C_i$ curve measurement

Assimilation rate (A) at various C_i for intact leaves was made with either the cup or the standard bottom half of the assimilation chamber. Photosynthesis and C_i became steady within 40–60 min after clamping on the leaf at an ambient CO_2 concentration (C_a) of around 400 μmol mol^{-1}. Thereafter, the photosynthesis response to varying C_a was measured. The C_a was lowered stepwise down to 30 μmol mol^{-1} and then returned to 400 μmol mol^{-1} to reestablish the initial steady-state value of photosynthesis. The C_a was then increased stepwise up to 1400–2000 μmol mol^{-1}. Measurements consisted of 8–10 measurements for each curve. When steady-state photosynthesis and C_i were achieved at each C_a, standard gas exchange parameters were determined.

Photosynthesis was measured at PPFD of 800 μmol m^{-2} s^{-1}, which was about 80–90 % of saturated A, to prevent photo-inhibition during often prolonged measurements. All measurements were carried out at a leaf temperature of 25°C and leaf to air vapor pressure difference (VPD) of 1.0–2.0 kPa, using a constant flow rate of 250 μmol s^{-1}. In the early morning, plants were taken from the glasshouse to the laboratory (room temperature of 25°C). There, plants were illuminated with fluorescent lamps that delivered PPFD of 150–400 μmol m^{-2} s^{-1} at leaf height. The plants were acclimated under the light at least 1 h before the measurements started.

Photosynthesis parameters

The equations for standard gas exchange parameters are essentially those derived by von Caemmerer and Farquhar (1981). CO_2 was fed to both surfaces of the leaf (free leaf) with the standard bottom chamber whereas CO_2 was fed only to the upper surface for the cup-attached leaf. Accordingly, $C_{i(c)}$ was determined for the free leaf while both $C_{i(c)}$ and $C_{i(m)}$ was obtained for the cup-attached leaf. For the latter configuration, we halved the boundary layer conductance, assuming that the boundary layer was symmetrically distributed between the two surfaces in the configuration with the standard bottom chamber.

RESULTS

CO_2 injection to the closed loop

With an aluminum foil clamped onto the cup, it was possible to test for leaks in the closed loop. At several s after a CO_2 injection a chromatographic peak appeared in both 1% and 5% CO_2 (Fig. 3). The CO_2 equilibrated at a higher concentration within 10–20 s after the injection and remained steady thereafter. The increase in CO_2 was fairly proportional to the concentration of added CO_2 (1:5), i.e, the CO_2 increased by 17 and 86 μmol mol^{-1} after 1% and 5% CO_2 injection, respectively. These results confirmed no apparent diffusion leaks through the closed loop.

Attaching the cup to the lower leaf surface inevitably restricts the CO_2 supply from one surface, and enlarges gradients of CO_2 inside the leaf depending on the intercellular conductance to CO_2. The effect of this restriction was detected by monitoring CO_2 depletion for $C_{i(m)}$ together with

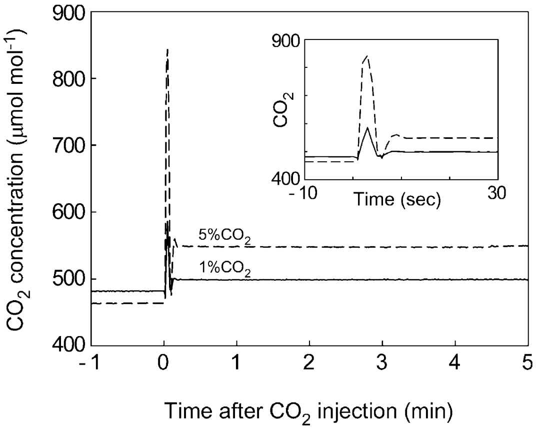

Fig. 3 Change in CO_2 in the closed loop after 0.2 ml of either 1 or 5% CO_2 was injected from the water seal of the condenser. The injection was done with aluminum foil clamped in place of the leaf. No sealing materials were used for this experiment. Data were scanned every 1 s. Note that the increase in CO_2 was proportional to the concentration of added CO_2 (1:5), i.e, the CO_2 increased by 17 and 86 μmol mol^{-1} after 1% and 5% CO_2 injection, respectively. Data are typical for three to five replications.

photosynthesis parameters when CO_2 was injected into the cup (Fig. 4). After a 5% CO_2 injection, A dropped solely because CO_2 diffused from the cup into the assimilation chamber. The stomata were open as indicated by the constant g_s of about 210 mmol m^{-2} s^{-1} (i.e. approximately 70% of the maximum g_s for the cup attached leaves) during the measurement. The A gradually recovered as $C_{i(m)}$ was depleted and returned to the original level around 15 min after the injection.

Leak test for open gas exchange system

We tested further for leaks in the open gas exchange system by coating the gaskets with several sealing materials over the range of the $A-C_i$ curve measurement (Fig. 5A). Any leak would appear as an apparent 'net photosynthesis'. The diffusion leak increased linearly as C_a increased, i.e., the gradient of CO_2 between inside and outside of the chamber increased. The apparent 'net photosynthesis' reached up to 2.5 μmol m^{-2} s^{-1} at the highest C_a (2000 μmol mol^{-1}). The similarity in responses regardless of the coating and the kind of sealing materials suggested either no effects of the coating or the absence of the leak from the sealed part (i.e. the gaskets). Accordingly, no coating material was used in all the subsequent experiments.

The effects of clamping the leaf and attaching the cup were tested (Fig. 5B) and gave comparable responses among the chamber with the aluminum foil, the killed leaf (i.e. photosynthetically inactive leaf) and the empty chamber (Fig. 5A, B). For the chamber monitoring the cup, the aluminum foil was expected to halve the diffusion leak through the gaskets. Nevertheless, the apparent 'net photosynthesis' was reduced only slightly (Fig. 5B). This suggested that the gaskets did not account for the observed leak. Finally, we determined K_{CO2} and K_{H2O} by creating either negative or positive concentration gradients of CO_2 and H_2O between inside and outside of the chamber (Fig. 6). An empty chamber with the standard bottom half was used for this experiment because the leak was not affected by cup-attachment or the clamped leaf (Fig. 5). No matter whether the gradient was inwardly or outwardly directed, the same leakage occurred (Fig. 6). K_{CO2} and K_{H2O} had mean values ranging from 0.17–0.24 μmol s^{-1} and 1.5–2.3 μmol s^{-1}, respectively. Average values of 0.21 μmol s^{-1} for K_{CO2} and 2.0 μmol s^{-1} for K_{H2O} were used for leak corrections in the subsequent $A-C_i$ curve measurements.

$A-C_i$ curve

The C_i measuring system gave continuous results while other features of gas exchange were monitored.

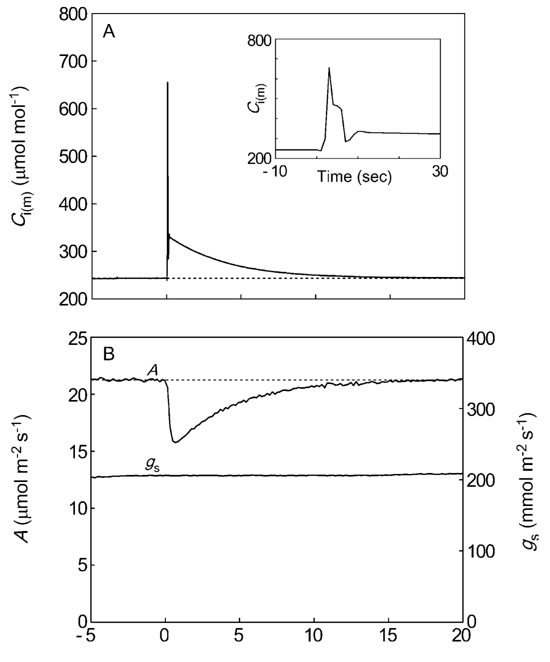

Fig. 4 Change in (A) internal CO_2 measured directly ($C_{i(m)}$) and (B) assimilation rate (A) and stomatal conductance to CO_2 (g_s) after 0.2 ml of 5% CO_2 was injected from the water seal of the condenser. The injection was done with an intact sunflower leaf. No sealing materials were used for this experiment. The broken lines indicate the original value right before the injection. The $C_{i(m)}$ was scanned every 1 s by LI-840A (LI-COR) whereas the gas exchange parameters were scanned every 10 s by LI-6400XT (LI-COR). The leaf was acclimated at ambient CO_2 under the normal measurement condition (see MATERIALS AND METHODS). Data are typical for four replications.

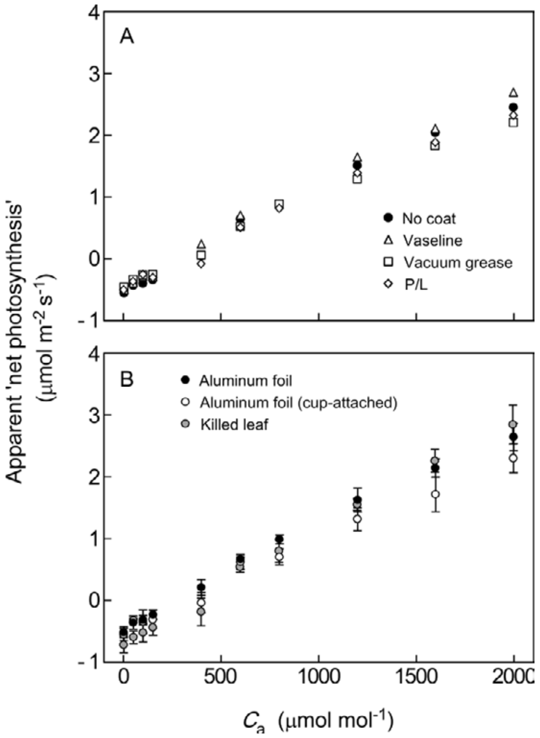

Fig. 5 (A) Effect of coating gaskets with several sealing materials on response of diffusion leak (apparent 'net photosynthesis') to various CO_2 concentrations (C_a) in the empty 6400-40 (LI-COR) chamber. Values are the average of three to four replicate curves. Each sealing material was thinly spread on every aspect of the gaskets. (B) Effect of clamping aluminum foil or killed leaf (i.e. photosynthetically inactive leaf) on the leakage. The effect of cup attachment was also tested with the aluminum foil clamped by the cup-attached chamber. Each measurement was done without coating the gaskets. Values are the average ± 1 SD of four to five replicate curves.

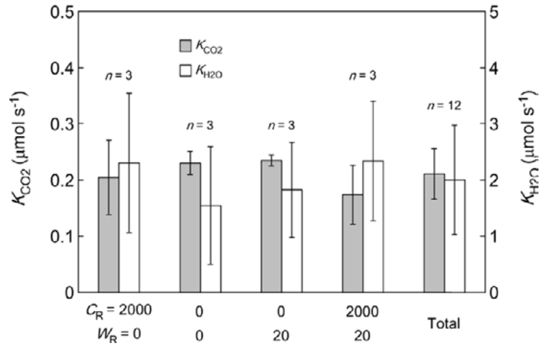

Fig. 6 CO_2 (K_{CO2}) and water vapor (K_{H2O}) molar flow rates caused by diffusion leaks for the empty LI-6400-40 chamber. Each K_{CO2} and K_{H2O} was determined simultaneously for four different combinations of reference CO_2 (C_R) and reference water vapor (W_R): from the left, high C_R (2000 μmol mol^{-1}) and low W_R (0 mmol mol^{-1}), low C_R (0 μmol mol^{-1}) and low W_R, low C_R and high W_R (20 mmol mol^{-1}), high C_R and high W_R, and averaged values for the whole. Values are the average ± 1 SD. The total averages for K_{CO2} and K_{H2O} were 0.21 ± 0.05 μmol s^{-1} ($n=12$) and 2.0 μmol s^{-1} ± 1.0 μmol s^{-1} ($n=12$), respectively.

Typical direct C_i measurement cycle during $A-C_i$ curve measurement is shown in Fig. 7. After clamping on the leaf, at an ambient CO_2 concentration (C_a) of around 400 μmol mol^{-1}, photosynthesis and $C_{i(m)}$ became steady within 40–60 min depending on the leaf. During this time, water vapor concentration in the closed loop also became steady at around 26 mmol mol^{-1}, i.e, somewhat lower than saturated humidity at the room temperature (25°C). In general, the $C_{i(m)}$ and $C_{i(c)}$ followed the change in CO_2 concentration in a similar manner. When C_a changed stepwise, steady-state photosynthesis and $C_{i(m)}$ were achieved within 10–20 min as we observed with the injection test (Fig. 3). Mostly, the $C_{i(m)}$ became steady as fast as the $C_{i(c)}$. We conducted the 'match' of gas analyzer calibrations at each C_a step after the steady-state photosynthesis before moving to the next step. A slight increase in the $C_{i(m)}$ by approximately 2 μmol mol^{-1} was observed during ca. 30 s match mode, possibly associated with either altered flow rate/pressure or bulk air movement through the leaf during the mode. After coming back from the mode, the $C_{i(m)}$ decreased to the steady value again within a minute. Accordingly, the data were taken a little while after the match was completed so that the potential bulk air movement was neglected.

At an ambient CO_2 of 400 μmol mol^{-1}, the average $C_{i(m)}$ was 268 ± 13 μmol mol^{-1}, and was lower than the $C_{i(c)}$ by, on average, 10 μmol mol^{-1}. The difference ($C_{i(c)} - C_{i(m)}$) became smaller as the C_i decreased (inset of Fig. 7). The $C_{i(m)}$ versus $C_{i(c)}$ relationship corresponding to Fig. 7 and shown in Fig. 8 indicates that the slope of the relationship was slightly but consistently larger than 1. The leak correction hardly affected this relationship. These results probably reflect changes in the internal gradient when the cup was attached (Sharkey et al., 1982; Parkhurst et al., 1988; Parkhurst and Mott, 1990). To see this effect on an $A-C_i$

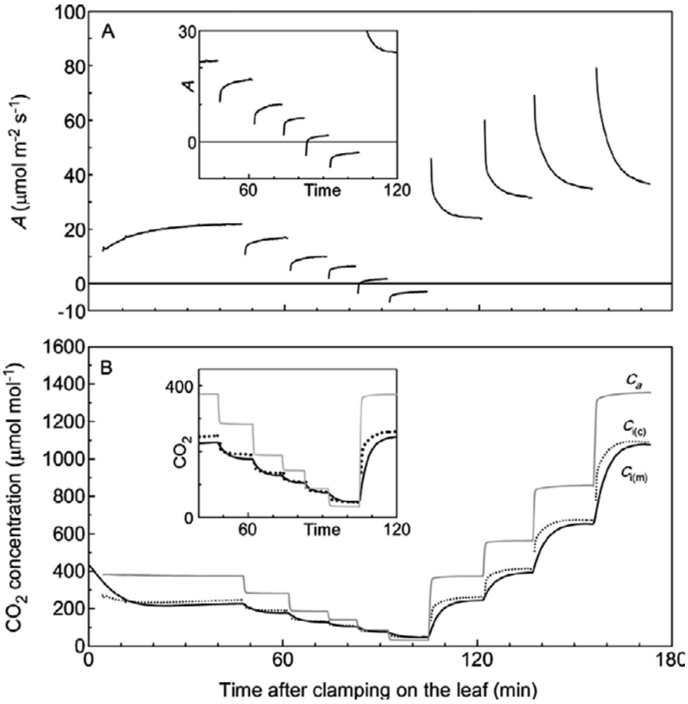

Fig. 7 Response of (A) assimilation rate (A) to (B) various internal CO_2 (C_i) for a cup-attached leaf. The C_i was derived from direct measurement ($C_{i(m)}$) and calculation ($C_{i(c)}$). Change in the ambient CO_2 concentration (C_a) is also shown with the C_i. Between each steady-state photosynthesis the two IRGAs inside the chamber head were matched with one another. The A and $C_{i(c)}$ were corrected by offsetting the difference of C_a at each match but not corrected for the diffusion leakage. For example, C_a was calibrated as −10 μ mol mol^{-1} at C_a of 1400 μmol mol^{-1}. Then, the data were corrected with the calibrated C_a throughout, and the correction was repeated at each C_a. In $C_{i(c)}$, the data for about one min right after the change in C_a were removed due to the extreme values. Data are typical for six replications.

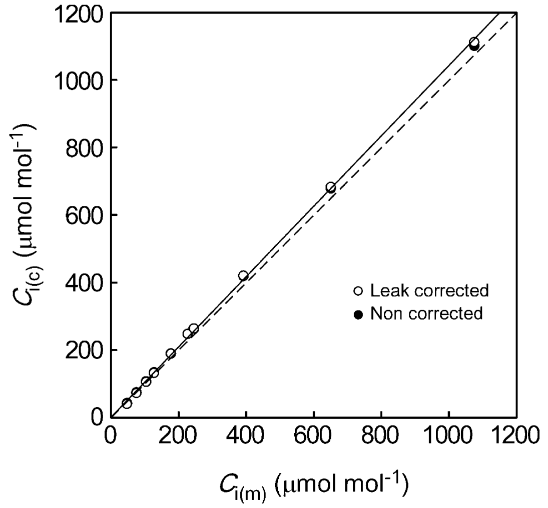

Fig. 8 Relationship between measured ($C_{i(m)}$) and estimated ($C_{i(c)}$) internal CO_2 for the cup-attached leaf shown in Fig. 7. Data were either corrected or not for diffusion leak by using the average K_{CO2} and K_{H2O} determined in the former experiment (Fig. 6). A regression line obtained for the leak-corrected data is shown as a solid line, whereas a 1:1 line is shown as a dashed line. Representative experiment from six replications.

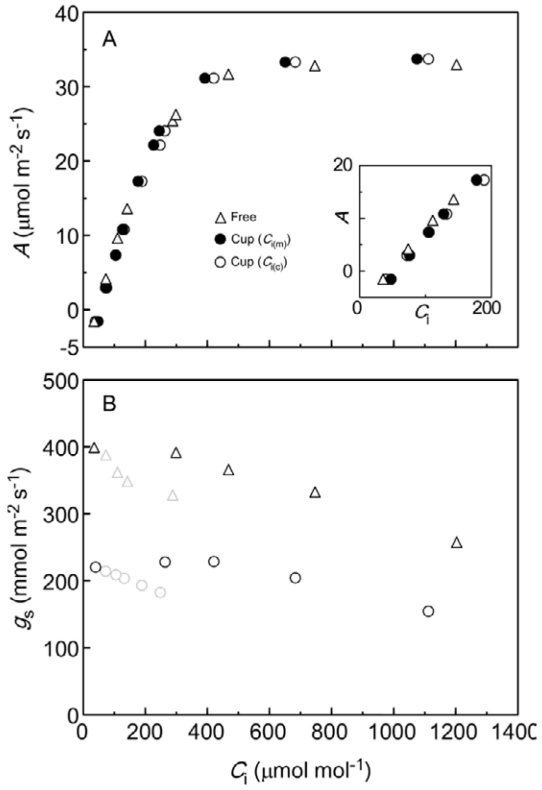

Fig. 9 (A) Response of assimilation rate (A) and (B) stomatal conductance (g_s) to various C_i shown in Fig. 7. Measured ($C_{i(m)}$) and calculated ($C_{i(c)}$) internal CO_2 are indicated for the cup-attached leaf, and the response for the free leaf was also determined for the same leaf following the measurement with the cup. Data were corrected for diffusion leak using the average K_{CO2} and K_{H2O} determined in the former experiment (Fig. 6). Note that the stomata responded to the change in CO_2 concentration in a particular fashion for both the free leaf and the cup-attached leaf. The g_s increased (shown as symbols pale in color) during decreasing CO_2 concentration (Fig. 7), and reached its maximum at the lowest C_a of 30 μmol mol^{-1}. Then, the g_s remained high when the CO_2 retuned to the ambient C_a of 400 μmol mol^{-1}, and declined with increasing C_a above the ambient. The representative experiment from three replications.

curve, we compared these data with the curve for a free leaf with open stomata (Fig. 9A). The $A-C_i$ curves obtained in the same leaf were substantially the same regardless of cup attachment. The C_i was always lower at each comparable C_a step in the cup-attached leaves than in the free leaves because of a reduction in g_s, resulting in the lower A especially in the lower C_i region (Fig. 9B). For the cup-attached leaf, the maximum g_s (often observed at the lowest C_a) ranged from 229 to 314 mmol m^{-2} s^{-1} with an average of 285 mmol m^{-2} s^{-1} which was approximately 70% of that in the free leaves with open stomata.

DISCUSSION

The entire system constructed here resembled the one developed by Sharkey et al. (1982) but incorporated the direct measurement of C_i (i.e. the closed loop) from Boyer and Kawamitsu (2011). One of the critical features of this system was the micro blower which constantly circulated the air in the loop with minimal pressure. This avoided pulses that might cause variation or bulk air flow through the leaf. Lauer and Boyer (1992) used fluid movement in the loop to circulate the air smoothly but did not measure gas exchange by the leaf. In the present work, the smooth and continuous air movement in the loop led to stable and fast responses in the equilibrated CO_2. This helped to retain the fast response and environmental control of the LI-6400 system. Although a slight change in the $C_{i(m)}$ occurred during the match mode the effect can be simply avoided by waiting extra minutes for the $C_{i(m)}$ to recover.

Effects of leaks

As the system depended on the accurate measurement of trace gases, diffusion leaks could be problematic whenever pressure or CO_2 concentration gradients existed between the inside and outside of the chamber or closed loop. The effect may become large as the chamber size decreases because leaks are a larger fraction of the measured photosynthesis as projected leaf area decreases (LI-COR, 2008). The C_i measured directly was little affected by leakage as indicated by the CO_2 injection. By keeping the projected leaf area small (2 cm^2) our system had a smaller cup /loop volume than in previous systems (Sharkey et al., 1982; Lauer and Boyer, 1992; Boyer and Kawamitsu, 2011). This may have helped to prevent leakage while maintaining a response as fast as the similar system measuring a larger leaf area (Boyer and Kawamitsu, 2011).

For the open gas exchange system, the diffusion leak of CO_2 was readily detected as the apparent 'net photosynthesis' at various CO_2 concentrations (Flexas et al., 2007). Unexpectedly, there was only a marginal effect on leakage caused by the isolation of the cup from the open gas exchange flow. Assuming that the leakage was larger at the interface between the gaskets than through the gasket itself (Flexas et al., 2007), it may be reasonable that the leakage remained when CO_2 diffused from the interface between the upper gasket and the aluminum foil instead of the lower gasket. However, coating the interface of the gaskets with various materials did not diminish the leaks. The maximum or minimum apparent 'net photosynthesis' with the empty chamber was almost half of the one found by Flexas et al. (2007), suggesting that the basal leakage was relatively small in our experiment. This was also supported by the lower K_{CO2} and K_{H2O} found in this experiment than in Rodeghiero et al. (2007) with a comparable setup. Accordingly, we suspect the leakage in this experiment arose somewhere other than the gasket. However, the leakage was not negligible and could not be eliminated (Fig. 5). Therefore, it was necessary to estimate leakage and correct $A-C_i$ data in this open gas exchange system (Flexas et al., 2007; Rodeghiero et al., 2007).

There was a reasonably close agreement between the measured and calculated C_i as was confirmed in the previous study by Sharkey et al. (1982). The correction for the leakage hardly affected the relationship between the two, suggesting that the estimated leakage had little influence on the calculated C_i over the entire range of CO_2.

Effects of cup attachment on CO_2 environment in leaves

The measurement of C_i was tested in sunflower leaves having stomata on the both surfaces (i.e. amphistomatous leaves). Attaching the cup on the lower surface inevitably caused a decrease in g_s although the effect may not be the same for each surface (g_s was maintained at 70% of the free leaf). It may be associated with the g_s ratio (upper/ lower) for the two surfaces (Mott and O'Leary, 1984) and/ or stomatal adjustment to cup attachment observed in sunflower leaves (Boyer and Kawamitsu, 2011).

The cup-attached leaf should increase the CO_2 gradients in the leaf because it doubles the diffusion path of the free leaf (Parkhurst et al., 1988; Parkhurst and Mott, 1990; Boyer and Kawamitsu, 2011). It seems likely that the gradient or intercellular conductance would limit photosynthesis in leaves which have stomata only on one surface (i.e. hypostomatous leaves) (Parkhurst and Mott, 1990; Evans and Loreto, 2000). We technically altered the amphi- to hypostomatous leaves by attaching the cup on one surface. The slightly but consistently lower $C_{i(m)}$ than $C_{i(c)}$ may be evidence of the finite intercellular conductance to CO_2. In return, one can briefly estimate the intercellular conductance by solving $0.5A/(C_{i(c)}-C_{i(m)})$, according to Sharkey et al. (1982). In this study, the ambient data (i.e. $A=25$ μmol m^{-2} s^{-1} and $C_{i(c)}-C_{i(m)}=10$ μmol mol^{-1}) estimate the intercellular conductance to be approximately 1.2 mol m^{-2} s^{-1}, a value similar to 1 mol m^{-2} s^{-1} determined in *X. strumarium* (Sharkey et al., 1982). These values are 4-5X greater than the stomatal conductance and, in effect, probably too large to be detected as a measurable difference in the $A-C_i$ curve as was seen in Fig. 9A and ones in other amphistomatous species (Mott and O'Leary, 1984; Parkhurst and Mott, 1990). Furthermore, the gap between the $C_{i(m)}$ and the $C_{i(c)}$ became a few μmol mol^{-1} as the CO_2 concentration decreased whereas the gap becomes less influential on A as substrate CO_2 becomes saturated at the site of carboxylation (see. Fig. 9). This may imply small effects of the gradient on $A-C_i$ curve measurement for the leaves with high intercellular conductance. On the other hand, the large effect should be readily detected by the gap with the direct measurement, and the estimated intercellular conductance will be used for calculating average C_i in leaves, if needed.

Detecting internal CO_2 directly

Like other systems for measuring C_i directly (Sharkey et al., 1982; Mott and O'Leary, 1984; Lauer and Boyer, 1992; Boyer and Kawamitsu, 2011), our system is only applicable to amphistomatous leaves but not to hypostomatous leaves because the system blocks CO_2 through one surface. Despite this constraint, the direct measurement of internal CO_2 system may facilitate the $A-C_i$ measurement especially when stomatal closure brings about the uncertainty in calculation of C_i because the directly measured C_i is free from the assumptions needed for the calculations (Boyer and Kawamitsu, 2011). The uncertainty especially arises from the patchy stomatal closure (Terashima et al., 1988) or the cuticle where the ratio of diffusivities for water vapor and CO_2 differs from stomatal one (Boyer et al., 1997). Given patchy stomatal closure, internal CO_2 may not be distributed uniformly due to the limited lateral diffusion, and the calculated C_i can be no longer reliable (Terashima et al., 1988; Terashima, 1992). With closed stomata, cuticle still allows water to move across but not CO_2 as much, which has a large impact on calculating C_i (Boyer et al., 1997). In either case, the C_i relying on water vapor is potentially overestimated, tracing apparent non-stomatal limitation on photosynthesis with the $A-C_i$ curve (Terashima et al., 1988; Mott, 1995; Buckley et al., 1997; Meyer and Genty, 1998). As for internal CO_2 gradient, these unappreciated players in the calculation are expected to be detected in the difference between measured and calculated C_i for leaves when stomata close.

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